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14. ABSTRACT
The purpose of this study is to test whether mechanical stimulation of a co-cultured biomaterial scaffold can improve/expedite healing of a tendon-to-bone interface for soft tissue repair. There are several precursor milestones that need to be achieved before the purpose can be tested. Namely, these milestones include: determining an appropriate scaffold for the tendon to bone interface, characterizing the co-culture behavior on the selected scaffold, developing a mechanical bioreactor to stimulate co-cultured scaffolds, and characterize the effect of mechanical stimulation on the co-cultured scaffolds. Currently, techniques have been established to condition the scaffolds by adsorbing attachments proteins found in fetal bovine serum. Efficient protocols for extracellular matrix digestion and analysis have been developed to save scaffold materials and correlate data. Finally, a customizable bioreactor was designed to selectively mechanically stimulate tendon-to-bone tissue engineering co-cultured scaffolds.

15. SUBJECT TERMS
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INTRODUCTION

Tissue engineering techniques have been well established for single tissue types[1-6]. Interface tissue engineering is being researched more frequently[7,8]. One specific area of interface engineering is tendon-to-bone interfaces, called enthesis[9-12]. The purpose of this study is to test whether mechanical stimulation of a co-cultured biomaterial scaffold can improve/expedite healing of a tendon to bone interface for soft tissue repair. There are several precursor milestones that need to be achieved before the purpose can be tested. Namely, these milestones include: determining an appropriate scaffold for the tendon to bone interface, characterizing the co-culture behavior on the selected scaffold, developing a mechanical bioreactor to stimulate co-cultured scaffolds, and characterize the effect of mechanical stimulation on the co-cultured scaffolds. The final test will be validated in a tendon to bone animal model. These milestones are outlined and described in this report.

BODY

A forward on the organization of the section: body. The body will be organized based on the approved specific aims from the original grant. The specific aim will be repeated verbatim from the grant and then a brief methods description, followed by relevant data and discussion pertaining to the specific aim will be presented.

Specific Aim #1 : Preparation of the candidate scaffold materials, including non-degradable woven polyester fabric degradable woven PLA fabric, and biologic collagen scaffold for cell adhesion and proliferation

Task 1: Coat the scaffolding materials with fibronectin and/or collagen (months 0-2)

1.a coat with human fibronectin and/or collagen (Quarter 1, University of Memphis)

1.b confirm protein adsorption (Quarter 1, Antibody labeling of proteins, University of Memphis)

As communicated in the previous annual report, **Specific Aim 1 has been completed.** Briefly, the conclusions for specific aim 1 were that neither human fibronectin nor collagen coatings induced higher cell numbers than no coating. Second, soaking the scaffolds in fetal bovine serum prior to cell seeding is the preferred method to condition the scaffolds for cell culture and will be used for the remaining cell culture studies.

Specific Aim #2: Optimize the tissue-specific coatings using osteoblasts and fibroblasts in co-culture on the available scaffolding materials. Narrow the scaffolding materials to one selection

Task 2: ECM coating on scaffolds (months 1-6)

2.a. Seed osteoblasts on scaffolds for 7, 14, 21, and 28 days (Quarters 1-2, University of Memphis)

2.b. Seed fibroblasts on scaffolds for 7, 14, 21 and 28 days (Quarter 1-2, University of Memphis)

Task 2 was completed and reported in the previous annual update. Briefly, mechanical testing of scaffolds (not initially specified in the original grant), Live/Dead staining over 28 days and scanning electron microscopy (SEM) was performed. Mechanical testing determined that the PLA fabric is the best candidate based on degradability and good tensile properties. SEM showed cells attached and deposited matrix on the scaffolds. Live/Dead staining showed good cell viability over the 28 day study for all scaffolds.

Task 3: Characterization of ECM coated scaffolds (months 3-6)

3.a. Cell attachment studies (Quarter 2, University of Memphis)

As reported in the last update this Task 3.a was completed. The FBS coated scaffolds had higher cell attachment than the fibronectin coated scaffolds. This data in conjunction with the live/dead fluorescent imaging shows that scaffolds coated with FBS provides a good attachment site for the cells.

Task 3.b. Cell proliferation studies (Quarter 2, University of Memphis)

Task 3.c. ECM composition studies total collagen and GAGs (Quarter 2, University of Memphis)

Task 3 was completed and reported in the previous April 2012 annual update.

Task 4: Tissue selective ECM coating on scaffolds (months 6-9)

4.a. Seed osteoblasts and fibroblasts in co-culture for 7, 14, 21, and 28 days (Quarter 3, University of Memphis)

4.b. Characterization of Co-cultured scaffolds, repeat 3.a, 3.b, and 3.c. (Quarter 3, University of Memphis)

To determine the appropriate co-culture media formulation with respect to mineralization, NIH 3T3 mouse fibroblasts (ATCC, Manassas, VA) or MC 3T3 mouse calvarial osteoblasts (ATCC, Manassas, VA) cells were seeded on tissue culture plastic (TCP) in single culture with varying concentrations of beta-glycerophosphate disodium pentahydrate (β-GP) (MPbio, Santa Ana, CA) to balance unimpeded osteoblast mineralization with low fibroblast

mineralization. Both cell types were seeded at $1x10^4$ cells/mm^2 per well in a 12-well plate(BD Falcon, San Jose, CA). The culture media formulation each cell type received was alpha-MEM (Hyclone, Waltham, MA) containing 10% fetal bovine serum (FBS) (Hyclone, Waltham, MA) + 1x Antibiotic/Antimycotic (Ab/Am)(Gibco, Grand Island, NY) and at every media change 25 μg/ml L-ascorbic acid (AA) (Acros Organics, NJ) was freshly added. To this formulation either 0, 1, 3, or 5 mM of β-GP was added. All groups at each timepoint were in triplicate. Media was changed every 2-3 days. Seeded plates were cultured in an incubator at 37 °C and 5% CO_2. At timepoints of 1, 4, 7, and 14 days, media was removed from the plates and samples were frozen until the end of the study, at which point 1 mL of biology grade water was added and all samples were dismembrated with an ultrasonic dismembrator (Fisher Scientific, Waltham, MA). Aliquots were then taken to perform picogreen assay for DNA quantification (Invitrogen, Grand Island, NY), Pierce BCA for total protein (ThermoScientific, Rockford, IL), and calcium assay for mineralization (Pointe Scientific, Canton, MI). All assays were performed according to the manufacturer's protocols. After the data were collected, total protein and mineralization data were normalized to DNA.

Figure 1 shows that the NIH 3T3 fibroblasts did not differ significantly in total protein per cell regardless of β-GP concentration. Even over time there was only a gradual but still significant increase in protein deposition between days 1 and 14. The MC 3T3 osteoblasts were also not consistently affected by the β-GP. At early timepoints of days 1 and 4 the increasing β-GP tended to increase protein deposition per cell but starting with day 7 to day 14 to opposite appeared to occur.

Overall, β-GP had little effect on total protein deposition which may be more strongly attributed to the ascorbic acid instead.

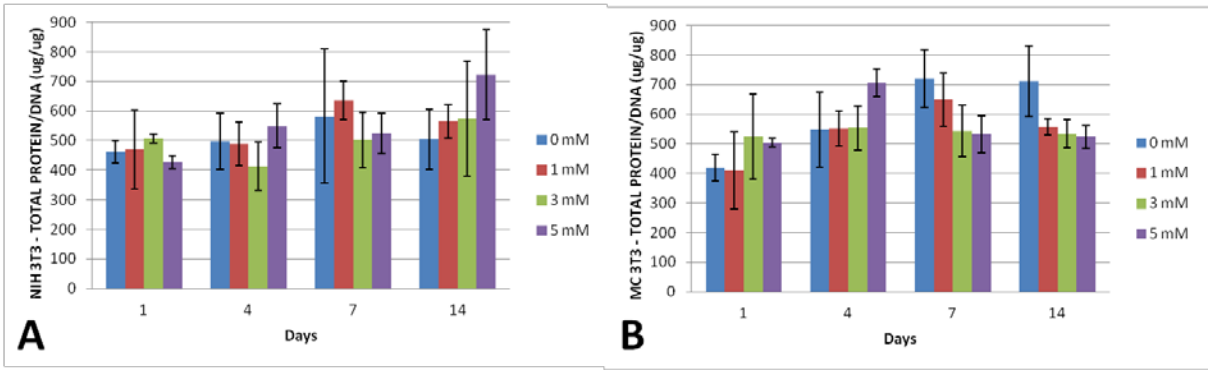

Figure 1. Total protein deposition of (A) NIH 3T3 fibroblasts and (B) MC 3T3 osteoblasts cultured in 0, 1, 3, or 5 mM β-GP. There was no significant difference in NIH 3T3 total protein with regard to β-GP amounts. Day 14 was significantly greater than day 1. For the MC 3T3 cells, there was no significant difference between the β-GP concentrations. All days (except between 7 and 14) were significantly different.

The effect of β-GP on mineralization through calcium deposition was more pronounced. In both cell types the β-GP concentration was a significant factor. Oddly, as seen in Figure 2, for NIH 3T3 cells, 1 mM β-GP had the highest amount of mineralization followed by 5 mM, 3mM, and 0mM, and all groups increased significantly with time. The MC 3T3 cells reacted more predictably in that with a higher concentration of β-GP there would be a higher amount of mineralization. At the 3mM concentration there was twice as much osteoblast mineralization than fibroblast mineralization at 14 days. The 3 mM β-GP concentration was used in media formulations for the subsequent studies.

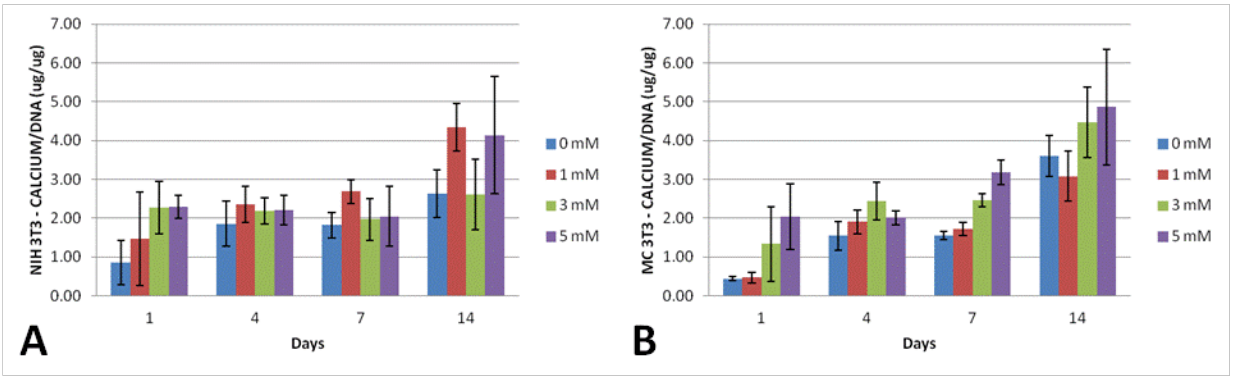

Figure 2. Calcium deposition of (A) NIH 3T3 fibroblasts and (B) MC 3T3 osteoblasts cultured in 0, 1, 3, or 5 mM β-GP. There was a significant increase in NIH 3T3 mineralization per cell with regard to β-GP concentrations of 1 mM and 5 mM. For MC 3T3s, all groups were significantly different except between 0 mM and 1 mM concentrations. For both cell types, mineralization significantly increased with time.

Scaffold Seeding

The scaffold used for this study is X-Repair®, a commercially available poly-l-lactic acid (PLA) woven fabric provided by Synthasome Inc, CA. X-Repair® is currently used for surgical reinforcement for tendon rotator cuff repair. All scaffolds in this study were seeded using this protocol. PLA fabric scaffolds were cut into strips with dimensions of 10 mm wide by 60 mm long. The edges were sealed by thermally fusing the polymer so the fabric structure would not unravel. The scaffolds were cleaned with a detergent solution, rinsed thoroughly with deionized water multiple times, and sanitized with 70% EtOH and UV light. Prior to cell seeding, the scaffolds were soaked in sterile culture medium containing FBS overnight to aid in cell attachment. The scaffolds were seeded with NIH 3T3 fibroblasts and MC 3T3 osteoblasts in co-culture at a $1x10^6$ cells/scaffold region, shown in Figure 3. The total number of cells per scaffold is $2x10^6$, one million each of fibroblasts and osteoblasts. All seeded scaffolds were grown in alpha-MEM containing 10% FBS +

1x Ab/Am + 3mM β-GP and at every media change 25 μg/ml AA was freshly added.

All seeded scaffolds were kept in an incubator at 37°C and 5% CO_2.

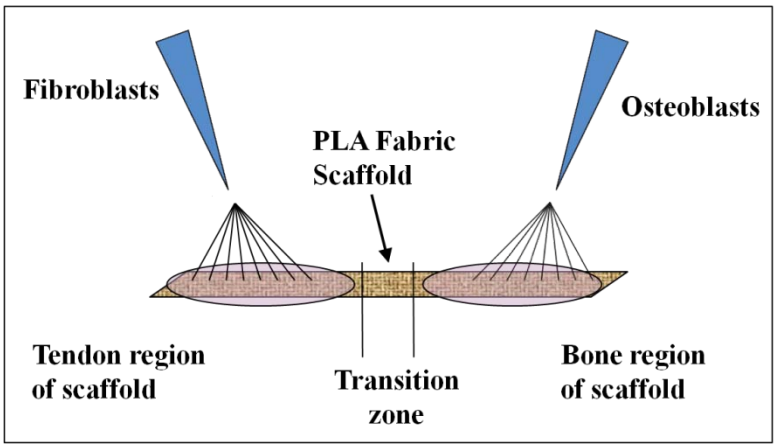

Figure 3. Schematic of how the PLA scaffolds are seeded with fibroblasts and osteoblasts on co-culture to make a tendon region and a bone region on the scaffold. There is a transition zone between the two regions where both cells interact.

Cell Tracking and Migration

To track how the cells were seeded and to confirm that tendon and bone regions can be seeded on a scaffold, the cells were labeled with two different fluorescent stains. Fibroblasts were labeled with Cell Tracker Green (Lonza, Alendale, NJ) and the osteoblasts were labeled with Cell Tracker Orange(Lonza, Alendale, NJ) according to the manufacturer's protocols. Labeled cells were seeded on the scaffolds as described previously, shown in Figure 4. The cells were allowed to attach to the scaffolds for 6 hours after seeding. The scaffolds were then placed in a sterile glass petri dish for fluorescence imaging. Using an inverted microscope (Nikon, Melville, NY) and a motorized stage (ASI Imaging, Eugene, OR) The entire scaffold was imaged by taking approximately 200 images at 4x magnification and stitching the images into a montage using BioQuant Osteo software(BioQuant, Nashville, TN).

Scaffolds were imaged at each of the tracker's specified wavelength then merged together with BioQuant. Images were taken at 6, 18, 30, and 42 hours for cell migration.

Both fibroblasts and osteoblasts are attached on their respective half of the scaffold creating the two tissue specific regions. Images were then taken every 12 hours afterwards to see if seeded scaffolds maintained this orientation. Figure 5 shows the montages over the 42 hour period. The fluorescence label loses intensity over time, and therefore, the exposure time was increased by the 42 hour image to intensify the colors on the scaffold. Because of the increased exposure, the 42 hour image has a noticeable amount of autofluorescence in the periphery of the image. Overall, there was not any noticeable migration on the macro scale at the border of the two different cell seeded regions.

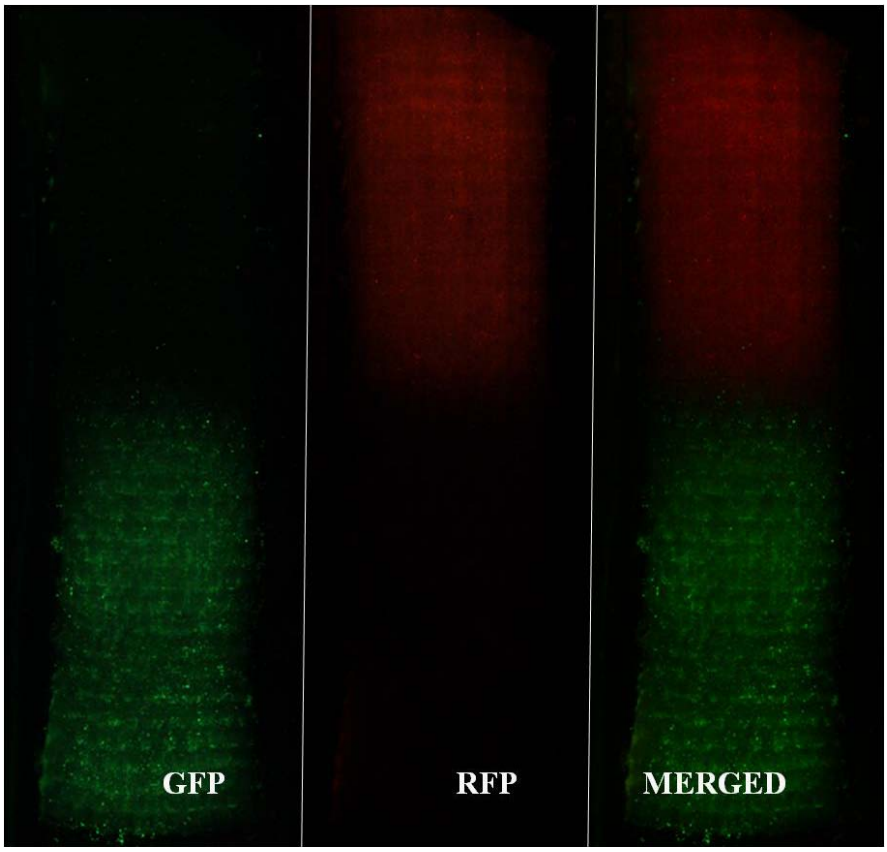

Figure 4: Fluorescent labeled fibroblasts (Green) and osteoblasts (Red) on the PLA scaffold form distinct tissue specific regions at 6 hours. The two images using the green fluorescent protein (GFP) and red fluorescent protein (RFP) filters were merged to show how the cells are seeded on the entire scaffold. Image consists of approximately 200 images at 4x magnification stitched together. Scaffold size is 10 mm wide by 60 mm long.

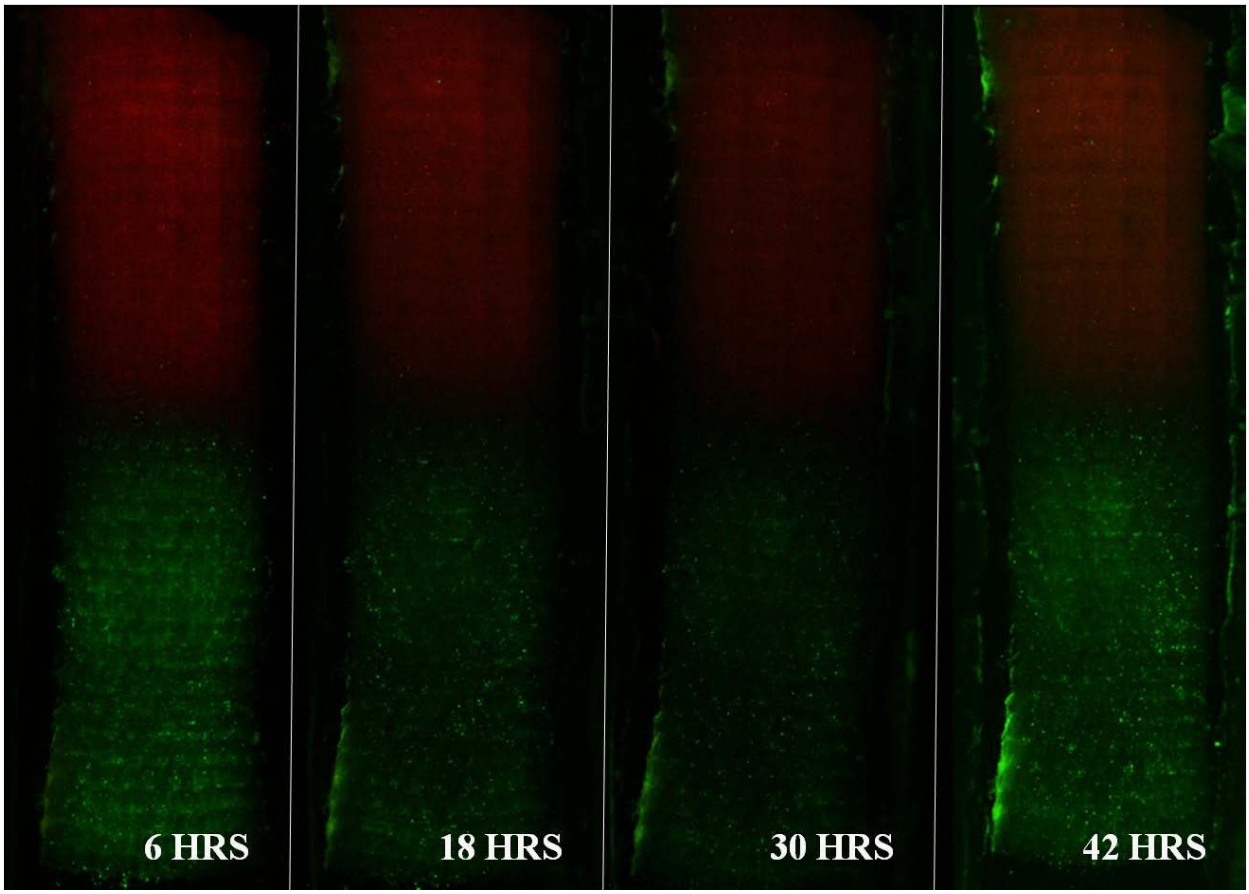

Figure 5: Merged fluorescent images over a 42 hour period. Images show that the cells maintain the tissue specific regions on the scaffold. No noticeable migration was observed in the measured time frame.

ECM deposition

Extracellular matrix deposition on the scaffolds was quantified for 28 days to access the coating applied to the scaffold. Cells were seeded as previously described. The culture medium used was alpha-MEM containing 10% FBS + 1x Ab/Am + 3mM β-GP and at every media change 25 µg/ml AA was freshly added. All scaffolds were cultured individually in 100mm non-treated polystyrene petri dishes. Scaffolds were moved to new petri dishes every 7 days to prevent cells that fell off the scaffolds to become confluent. Scaffolds (n=4) were collected at timepoints of 1, 7, 14, 21 and 28 days. At each timepoint, the 10 x 60 mm scaffolds were removed from

the petri dish and cut into three 10 x 20 mm sections. Each scaffold, therefore, produced a tendon section, a transition middle section, and a bone section for analysis. Each section was placed in a 1.5 ml microcentrifuge tube and 1 mL of a buffered enzymatic digestion solution of 100 ug/mL proteinase-K (Promega, Madison, WI) was added to every tube. All samples were then placed in an oven at 60°C overnight to digest the ECM. The following day phenylmethanesulfonylfluoride (PMSF) was added to a concentration of 5mM to inhibit the proteinase-K. All samples were homogenized using a sonic dismembrator and aliquots were taken from each sample to perform picogreen for DNA, alcian blue for glucosaminoglycan (GAG), and hydroxyproline (HYP) for collagen quantification. All volumes were carefully recorded monitored for normalization during analysis.

The last objective of this study was to quantify the basic components of tendon and bone ECM as it was deposited on the scaffolds. These components are GAG's and collagen measured by alcian blue and hydroxyproline, respectively. To show how matrix was distributed among the different regions the scaffold was cut into equal thirds creating a tendon, transition, and bone data point per scaffold. The DNA quantified in Figure 6, showed no significant differences between any particular sections indicating that cells were spread very uniformly over the scaffold including the transition section. DNA was used to normalize the GAG and HYP data. The alcian blue assay showed that GAG was significantly higher in the tendon region compared to the bone region and that the transition zone had a value between the two regions

for every timepoint, suggesting mixing contributions from each cell type. Lastly, there was no significant difference between scaffold regions for collagen deposition on the scaffolds. However, it was promising that collagen was continually deposited on the scaffolds for the duration of the 28 days study indication good cell viability and activity on the scaffold.

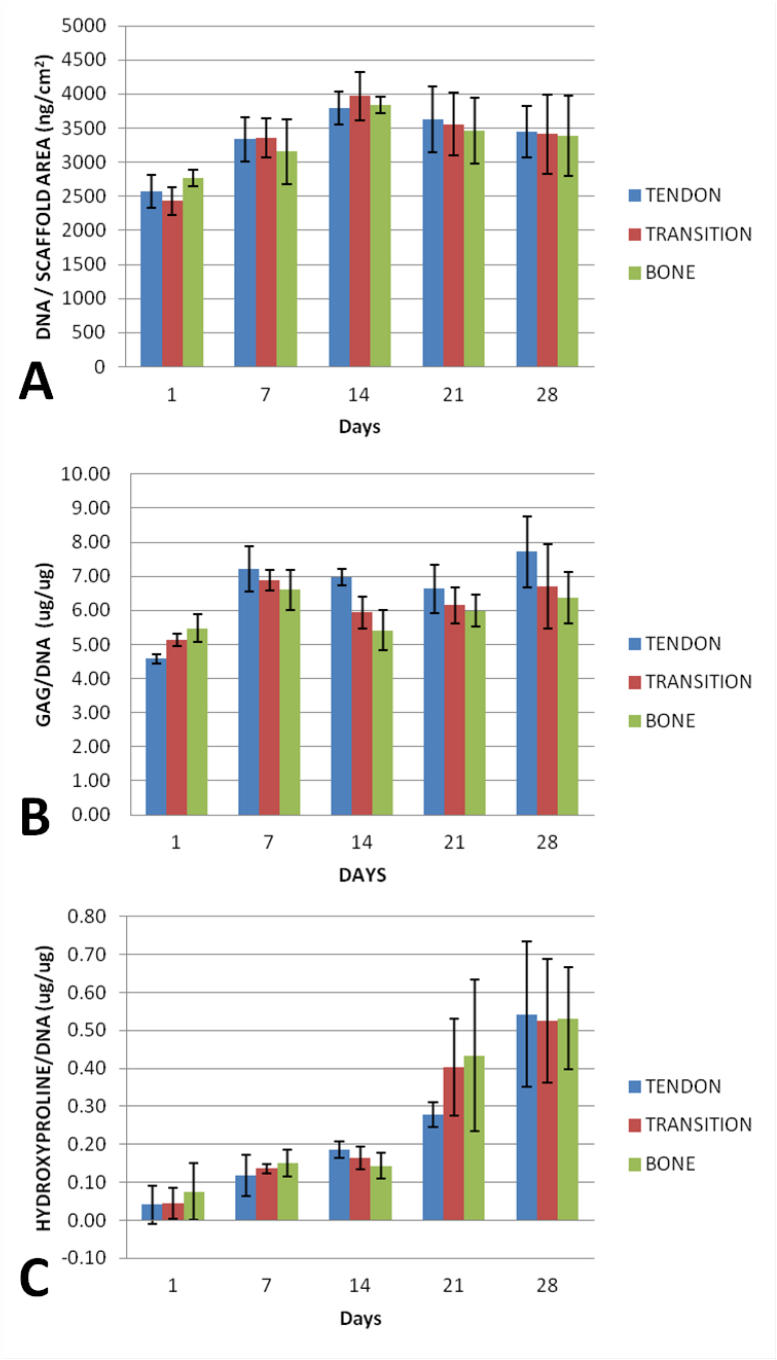

Figure 6: Quantification of DNA plus GAG and HYP deposition on the co-cultured scaffolds. The scaffolds were sectioned into equal thirds and analyzed separately. (A) DNA amounts were not significantly different between the scaffolds regions. (B) There was a significantly higher deposition for GAG/DNA in the tendon region compared to the bone region, with the transition region having values between the two. (C) There were no significant differences between groups for HYP/DNA deposition. However, continually increasing collagen deposition is promising in a co-culture model, indicating active and viable cells on the scaffold.

In conclusion for task 4, we selected a suitable co-culture media formulation for our initial work with an enthesis scaffold. We were able to seed the scaffold in co-culture to create two distinct tissue specific regions on the scaffold. Lastly, we measured ECM deposition on the two distinct regions plus a middle transition region.

Task 4 is completed. Expectations are these data will be published as the second publication for this project.

MILESTONE #1: Tissue Specific ECM coating on a scaffold (Month 9 - End of 3rd Quarter)

COMPLETED

Specific Aim #3: Enhance the optimized-tissue specific coating and scaffold by applying a cyclical tensile load during ECM deposition using a modified commercially available cell stretching system

Task 5: Mechanical stimulation of cell seeded scaffold (months 3-15)

5.a. Modify cell stretcher for chosen scaffolding material (Quarters 2-3, University of Memphis)

5.b. Seed cells on scaffold for 7, 14, 21, and 28 days with cyclic loading (Quarters 3-5, University of Memphis)

The first design of the bioreactor was built and initially there were constant problems with lack of sterility. The chamber could not be repeatedly autoclaved; thus, it was not getting sterile after each use. This introduced bacteria into each new test and caused many bacterial infections. To fix this, the chamber design was altered and rebuilt. The original

design used Weld-On 4 as a welding/bonding agent to create a watertight seal between the walls and base of the chamber. Two main design changes occurred. The first change involved removing the Weld-On and using clear 100% silicone as a weld/caulk and 316L stainless steel screws to join the walls to the base. This allows the screws to be used for structural integrity and the silicone to be used as a caulking and sealing agent. The entire chamber can now be repeatedly autoclaved. We have not had issues with contamination with the new design.

The second change involved replacing many of the polycarbonate clamp pieces with 316L stainless steel. The rational for this was that stainless steel is much stronger and much more resistant to deformations after numerous autoclave cycles. The new system setup is shown in Figure 7.

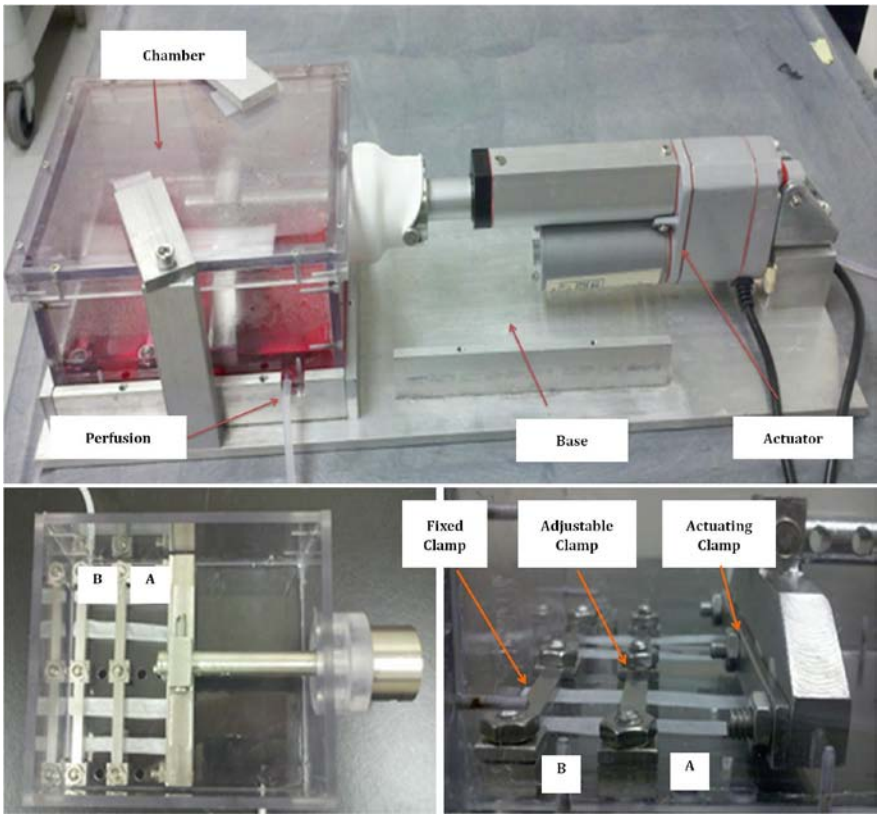

Figure 7. Custom bioreactor design used for our mechanical stimulation. The top image shoes the sterile chamber connected to the linear actuator with gas permeable media circulation lines attached. The bottom left image shows a top down view of the inside of the chamber with the actuator arm connected to the clamps that hold the scaffolds. The bottom left image shows the section labeled (A) between the actuating clamp and the center adjustable clamp receives mechanic stimulation and the section labeled (B) between the center adjustable clamp and the fixed clamp does not receive stimulations. The stretched region of the scaffold is placed in (A) and the unstretched seeded region is placed in (B). The entire chamber and actuator fits inside of the incubator at 37°C and 5% CO_2.

After solving the sterility issue, we began validating the bioreactor. Two groups of characterizations were performed: mechanical and biological. Mechanical characterization was performed to validate that the applied stretch was translated directly into experienced strain on the scaffolds. Polymeric solids and fabrics tend to exhibit viscoelastic properties in varying amounts so applied stretch does not always translate into experienced strain. In addition, the LabVIEW program was tested electronically (does the output match what it should) but was untested with scaffolds present.

For Mechanical validation , scaffolds were fixed into the bioreactor between the clamps. The LabVIEW program was set to stretch the scaffold at specified strain. Four visual markers were placed on each scaffold – two in the stretched zone, two in the non-stretched zone. A camera was used to take a picture with no stretch applied followed by a picture taken when the stretch was applied. A ruler was imaged in every picture to normalize pixels. This cycle was repeated 20 times for both fabric types, PET and PLA. A schematic of the two picture types is shown in Figure 8. Pixels from each image were counted and converted to inches by the using ruler as a standard. The relative change in distance is the experienced strain.

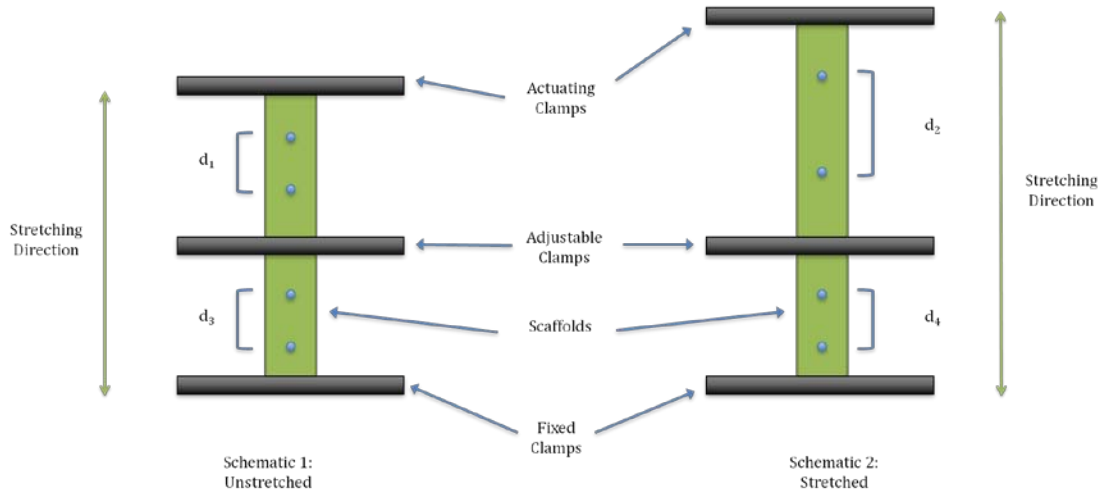

Figure 8: Schematic demonstrating how strain on the fabric scaffolds was determined. Reference markers were placed on the scaffold and measured. A sequence of images was taken at no strain and fully strained positions. Pixels were counted and normalized to a known distance.

Mechanical characterization was performed on PLA fabric at 5% strain and a PET fabric at 3% strain. Results showed that the unstretched portion of the PLA scaffold experienced

0.04±0.11% strain while the 5% stretched portion of the PLA scaffold experienced 4.96±0.32% strain. The resolution for the strain determination was 0.32%. In the PET scaffold, the unstretched portion of the scaffold experienced 0.02±0.18% strain while the 3% stretched portion experienced 2.97±0.13% strain as shown in Figure 9.

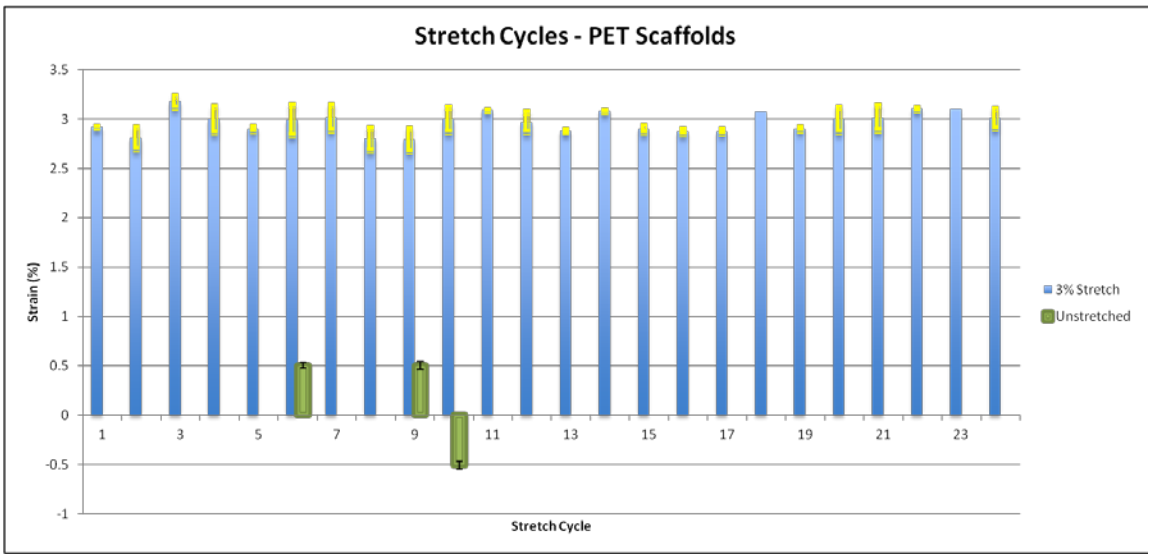

Figure 9: Stretch cycles of the PET scaffolds in the bioreactor. Each bar represents the average strain across all four scaffolds for that stretch cycle. Error bars represent one standard deviation.

Biological characterization was performed for two reasons; first, to validate the biological aspects of the bioreactor (i.e. is the bioreactor actually making a difference) and secondly, checking for uniform stretching and cell response across all scaffolds. PET scaffolds (6 scaffolds in bioreactor, 6 scaffolds in petri dish for control) were prepared using the protocol mentioned in Task 3, with NIH 3T3 fibroblasts. The scaffolds were strained for 14 days. Scaffolds were stretched 5% cycled at 1Hz for 1hr daily. To characterize the scaffolds, we are looking at cell viability (DNA, PicoGreen), GAG deposition in the matrix (Alcian Blue), and collagen deposition (hydroyproline). One scaffold from each group was removed for Live/Dead staining. Two-factor ANOVA was performed to look for uniformity along the length of the scaffold as well as between scaffolds in each group.

There was a significant increase in cell number between the control group and the experimental groups ($p<0.001$) shown in Figure 10. Cells were uniformly distributed along the length of the scaffolds in all groups as observed in Live/dead staining in Figure 11. GAG per cell was significantly reduced inside of the chamber for both the stretched and unstretched scaffolds when compared to the static control in the petri dish. Collagen deposition was significantly increased per cell on the scaffolds inside the chamber compared to the control. Further the stretched scaffold section had significantly more collagen deposition per cell than the unstretched scaffold section. This indicates that there is an effect of ECM deposition induced by the mechanical bioreactor.

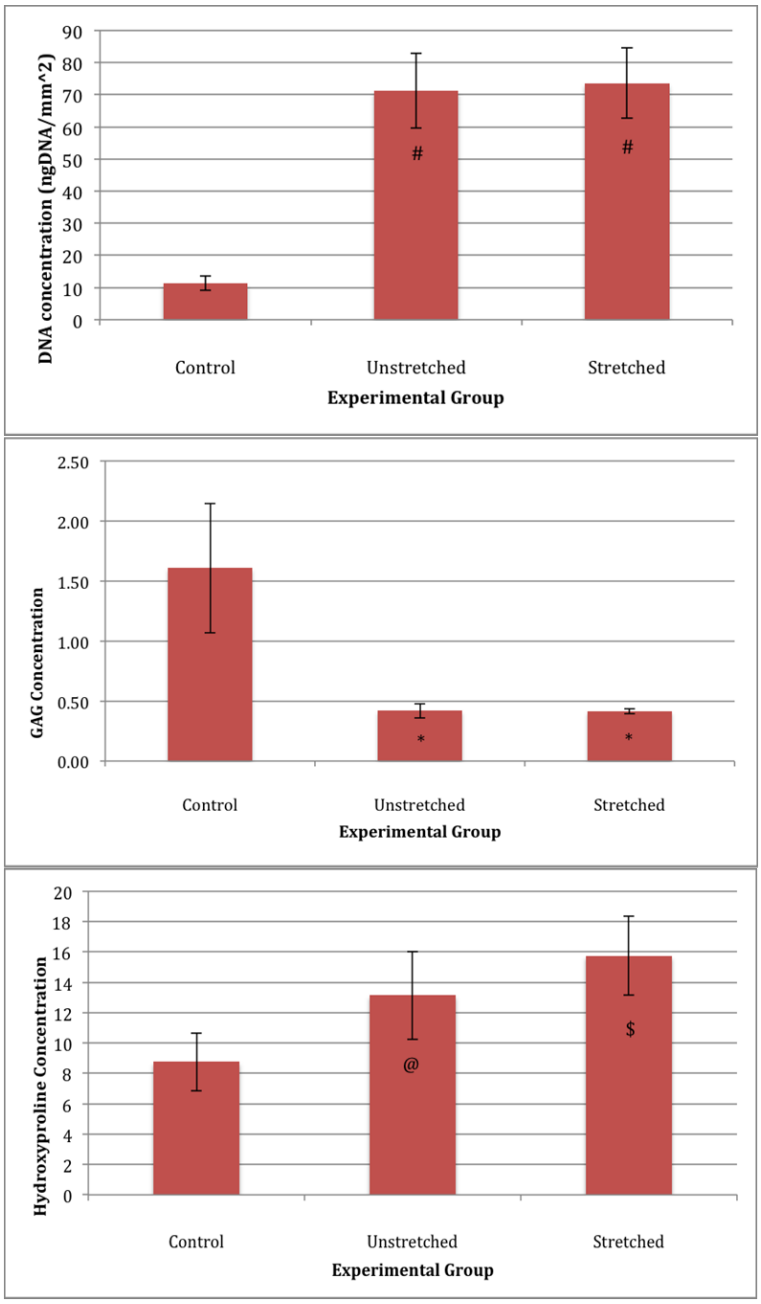

Figure 10: [TOP] DNA concentration by experimental group. n=5. #Statistically different than control ($p<1x10^{-6}$). [MIDDLE] GAG deposition normalized to DNA (ngGAG/ngDNA). n=5. *Statistically different than control ($p<0.001$) [BOTTOM]Hydroxyproline deposition normalized to DNA (ngHYP/ngDNA). n=5. @Statistically different than control ($p<0.05$) $ Statistically different than control ($p<0.01$)

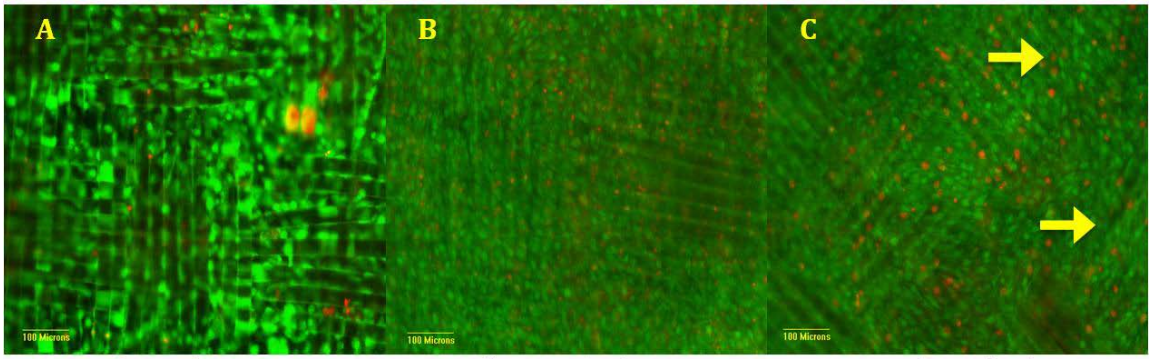

Figure 11: Representative images of Live/Dead stain on scaffolds. Green = living/viable cells; red = dead/non-viable cell. Arrows indicate some cell alignment on stretched portion of scaffolds.

Task 5 is completed. Expectations are these data will be published as the third publication for this project.

Task 6: Characterization of mechanically stimulated ECM coated scaffold (months 10-15)

6.a. Characterize scaffolds and ECM, repeat steps 3.a, 3.b, 3.c (Quarters 4-5, University of Memphis)

6.b *In vitro* Mechanical Tensile strength of ECM coated scaffolds (Quarter 5, University of Memphis)

Scaffolds were seeded with the same protocol as previously outlined in Task 4. After samples were seeded they were placed in the bioreactor pictured in Figure 7(see above). Two separate chambers were used each with 4 scaffolds per chamber. The fibroblast side of the scaffold was placed between the clamps labeled A and the osteoblast was placed in part B. One chamber had the adjustable center clamp

intact which stretched the A side but not the B (fibroblast stimulation but osteoblast static), termed Half-Stretched. These scaffolds were cultured for 21 days with the middle clamp intact then the clamp was removed to allow the cells to infiltrate the center region to deposit ECM and the scaffolds were stimulated for a further 14 days. In the other chamber the center clamp was never used which allowed for the entire scaffold to be stimulated for all 35 days, termed All-Stretched. We used a stretching regime of 5% cyclic strain at 0.5 Hz for 1 hour per day every day for the length study. The culture media formulation each cell type received was alpha-MEM (Hyclone, Waltham, MA) containing 10% fetal bovine serum (FBS) (Hyclone, Waltham, MA) + 1x Antibiotic/Antimycotic (Ab/Am)(Gibco, Grand Island, NY) + 3 mM of β-GP and biweekly 25 μg/ml L-ascorbic acid (AA) (Acros Organics, NJ) was freshly added. Media was completely refreshed every 7 days. Both chambers were kept in an incubator at 37 °C and 5% CO_2.

At the end of the 28 days the 10 x 80 mm scaffolds were removed from the chambers and cut into three equal sections. Each scaffold, therefore, produced a tendon section, a transition middle section, and a bone section for analysis. Scaffolds were decellularized to remove any fibroblasts or osteoblasts on the scaffolds prior to MSC seeding (See Task 10). The decellularization protocol was a mixture of freeze thaw, hypo- and hyper-ionic solutions. First samples were frozen at -80C, thawed and repeated. Then samples were alternated in solutions of deionized (DI) water for 1 hour and 10x phosphate buffered solution (PBS) for 1

hour. This cycle was repeated for 6 cycles, placed in DI water for one more hour, then soaked in 1x PBS for 1 hour prior to cell seeding.

The Matrix was digested as previously outlined and Picogreen (DNA), alcian blue (GAG), and hydroxyproline (Collagen) assays were performed. GAG and HYP data was normalized to DNA. The data presented in Figure 12 shows what was deposited on the scaffolds used for gene activation (described in Task 11). There was a significant difference between the all stretched and half stretched scaffolds for the DNA and GAG amounts. In the DNA data there is a drop in half stretched scaffold within the middle region. This is most likely due to the center clamp being there initially then in the remaining two weeks the cells didn't have time to fully fill in the gap. Overall there were generally more cells in the all stretched scaffolds. Fibroblasts deposited more ECM than the osteoblasts, and the middle region tended to mimic the trends produced by the fibroblasts. Stretching did not cause an increase in GAG or HYP deposition in the osteoblast region. Collagen deposition did increase in the all stretched fibroblast region. The ratio of collagen to GAG increased in the fibroblast and middle regions due to stretching but stayed unchanged in the osteoblast region. Overall, the all stretched scaffolds had increased cell number and fibroblast collagen to GAG deposition.

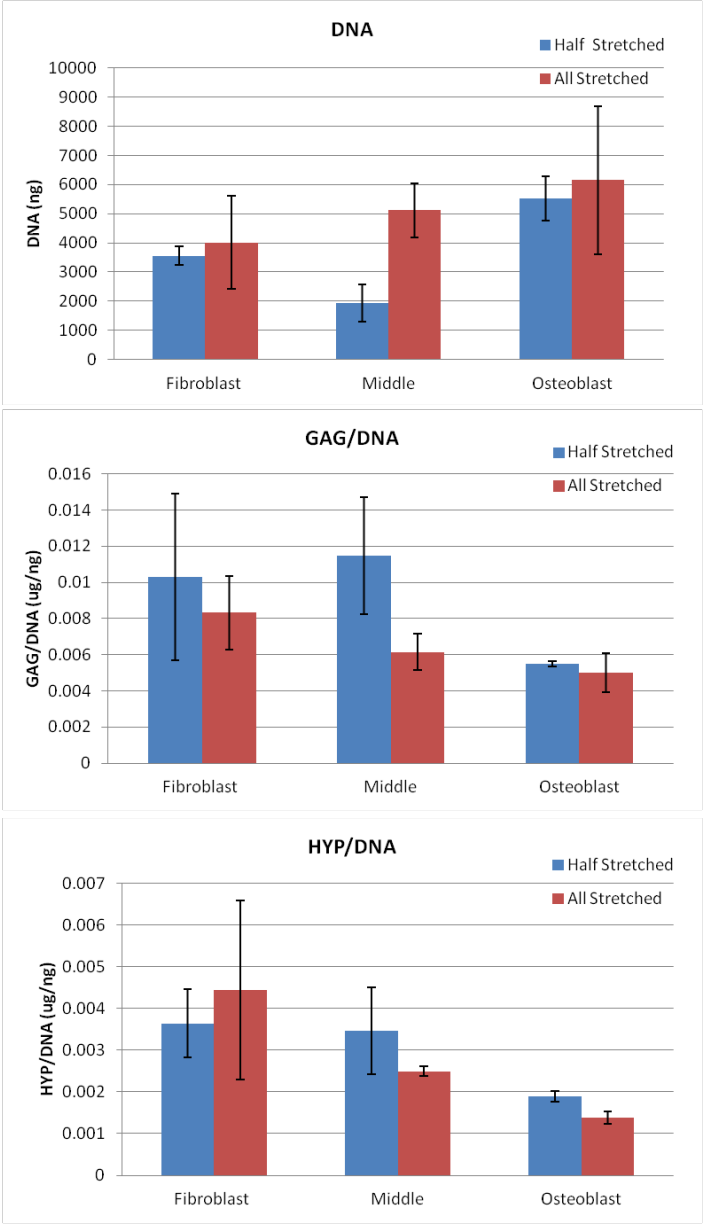

Figure 12. Characterization of ECM deposited on the scaffolds while in the mechanical bioreactor. Generally, the all stretched scaffolds had higher cell numbers than the partially stretched scaffolds. The fibroblast regions of the scaffold had higher deposition of ECM components and tended increase the amount of collagen deposited on the all stretched scaffolds compared to the partially stretched.

Synthasome Inc, graciously donated all PLA scaffolding materials for this project. However, the testing outlined in the original grant was a larger material commitment than Synthasome was able to provide. Unfortunately, due to lack of PLA scaffolding material donated from Synthasome, Inc. and time constraints of project time there was not enough samples to perform mechanical testing after culture in the bioreactor. With additional funding and time this data would be valuable to understanding the effects of culture and mechanical strains on the scaffolding construct prior to implantation.

Task 6 is completed. Expectations are these data will be published as the third publication for this project.

MILESTONE #2: Mechanically stimulated tissue specific ECM coated scaffold (month 15 End of 5th Quarter)

COMPLETED

Specific Aim #4: Evaluate the decellularized mechanically stimulated cytoselective tissue specific coating in a functional *in vivo* rabbit bone-tendon defect model.

The proof-of-principle in vitro study was performed as described in the April 2012 annual report. However, post operation complications with the surgery caused the study to be ended prematurely before relevant data could be acquired. Correspondence with IACUC and ACURO members was documented. The funding was not available to repeat the study, so a PRORP approved alternate specific aim was performed. The revised specific aim is listed below.

Revised Specific Aim #4: Evaluate the decellularized mechanically stimulated cytoselective tissue specific coating using mesenchymal stem cells (MSCs) in culture

Task 10: Deposition of ECM on scaffolds and mechanical stimulation of scaffolds

10.a Seed fibroblasts and osteoblasts on the scaffold in co-culture and stimulate during culture in the mechanical bioreactor for 4 weeks. (Quarter 6, University of Memphis)

10.b After culture, scaffolds are decellularized using a free thaw and mild detergent solution. (Quarter 6, University of Memphis)

10.c Decellularized scaffolds are then seeded with rat MSCs for 24 hours (Quarter 6, University of Memphis)

Tasks 10.a and 10.b are described in Task 6 as these tasks are procedurally related. Briefly, the scaffolds were seeded in co-culture and stimulated in the mechanical bioreactor for 5 weeks as previously described. These scaffolds with the deposited ECM coatings were decellularized and then re-seeded with rat mesenchymal stem cells (MSCs). MSCs were seeded on each scaffold section at $1x10^6$ cells per section. MSCs were also seeded on tissue culture plastic (TCP) as a control. Samples and cells were cultured in alpha-MEM containing 10% FBS + 1x Ab/Am for 24 hours. Then they were removed from the scaffolds for and prepared for gene activation analysis by real time polymerase chain reaction (rt-PCR).

This task has been COMPLETED.

Task 11: Analyze MSC gene activation with rt-PCR.

11.a After culture on scaffolds for 24 hrs cells are harvested and RNA is isolated. (Quarter 6, University of Memphis)

11.b Rt-PCR will be used to identify tissue specific markers for gene activation due to the ECM signaling of the tendon and bone side of the scaffold. (Quarter 6, University of Memphis)

After the 24 hour seeding time, scaffolds sections were placed in a sterile PCR grade 1.5 ml centrifuge tube and submerged in 1x-trypsin with EDTA for 5-10 mins to release MSCs. Scaffolds were removed, and centrifuged for 5 mins to form a pellet in the centrifuge tube. All liquid was aspirated, careful not disturb the pellet, the all tubes were immediately placed in liquid nitrogen to flash freeze all samples. RNA was then isolated using a Qiagen mini-kit according the supplier's protocol. RNA quality and quantity was measured using a q-bit analyzer. After analysis, amplification was deemed necessary. All RNA was transcribed to cDNA using Roche cDNA kit. Then applied biosciences TaqMan amplification kit was used according to the supplier's protocol to amplify the cDNA signal of the collected samples. Primers were designed for tissue specific genes listed in Table 1. After effective primers were identified, separate master mixes were then made with the primers for Collagen 3, Decorin, Osteocalcin, and Aggrecan. All primers were designed for rat genes and HGPRT was selected as the best reference gene. All samples plus master mixes were plated out and run for PCR in Roche LC480. After PCR was complete, data was analyzed for relative changes using the delta delta C_t method.

TABLE 1: Tissue-specific primers designed for MSC gene activation

GENE	TISSUE	ASSCESSION #	SEQUENCE	OUTCOME
Collagen I	TENDON/BONE	NM_053304.1	5′-catgttcagctttgtggacct-3′ [R] 5′-gcagctgacttcagggatgt-3′ [L]	Efficiency too low
Collagen II	FIBROCARTILAGE	NM_012929.1	5′-ccaggtcctgctggaaaa-3′ [R] 5′-cctctttctccggccttt-3′ [L]	Efficiency too low
Collagen III	TENDON/BONE	NM_032085.1	5′-tcccctggaatctgtgaatc-3′ [R] 5′-tgagtcgaattggggagaat-3′ [L]	DATA PRESENTED
DECORIN	TENDON	NM_024129.1	5′-ctccgagtggtgcagtgtt-3′ [R] 5′-gcaatgttgtgtcaggtgga-3′ [L]	DATA PRESENTED
SCLERAXIS	TENDON	NM_001130508.1	5′-cccaaacagatctgcacctt-3′ [R] 5′-tctgtcacggtctttgctca-3′ [L]	Efficiency too low
TENOMODULIN	TENDON	NM_022290.1	5′-tggatcaatcccactctaatagc-3′ [R] 5′-tcgctggtaggaaagtgaaga-3′ [L]	No PCR Binding
OSTEOCALCIN	BONE	M23637.1	5′-cattactgaccgctccttcc-3′ [R] 5′-cgcatagcctgtgattttca-3′ [L]	DATA PRESENTED
OSTEOPOINTIN	BONE	NM_012881.2	5′-cggtgaaagtggctgagttt-3′ [R] 5′-ggctacagcatctgagtgtttg-3′ [L]	Efficiency too low
ALKALINE PHOSPHATASE	BONE	NM_013059.1	5′-gcacaacatcaaggacatcg-3′ [R] 5′-tcagttctgttcttggggtacat-3′ [L]	Efficiency too low
AGGRECAN	FIBROCARTILAGE	NM_022190.1	5′-aatgggagccagcctacac-3′ [R] 5′-agaggcagagggactttcg-3′ [L]	DATA PRESENTED
COLLAGEN X	FIBROCARTILAGE	XM_001053056.3	5′-ccctattggaccaccaggta-3′ [R] 5′-actgcttggctgggaggt-3′ [L]	Efficiency too low
HGPRT	HOUSEKEEPING			REFERENCE

Figure 13 shows the relative MSC gene activation on the tendon, bone, and middle regions of the scaffold compared to the MSCs cultured on TCP. All genes were expressed more compared to the TCP control. However, there was no significant difference between any groups or any gene with the exception of the Fibroblast half stretched and the osteoblast all stretched. These data represents which genes were activated in the MSCs at 24 hours of culture on the ECM coated scaffolds. To be clear, these data do not represent how mechanical strain affected ECM deposited on the scaffolds, but how the MSC react to the mechanically stimulated ECM deposited

on the scaffolds. Generally the stretching had a larger impact on the osteoblast region compared to the fibroblast region because in both chambers the fibroblast region was exposed to mechanical stimulation. Stretching causes an increase in gene activation of the deposition of collagen, and a decrease in mineralization. Gene activation of mineralization was expressed less when stretched. Stretching also decreased the tendon specific gene activation in the osteoblasts but was higher when the osteoblast region was static. Gene activation of the cartilage specific aggrecan remains unchanged in the fibroblast region but was slightly decreased in the stretched osteoblast section.

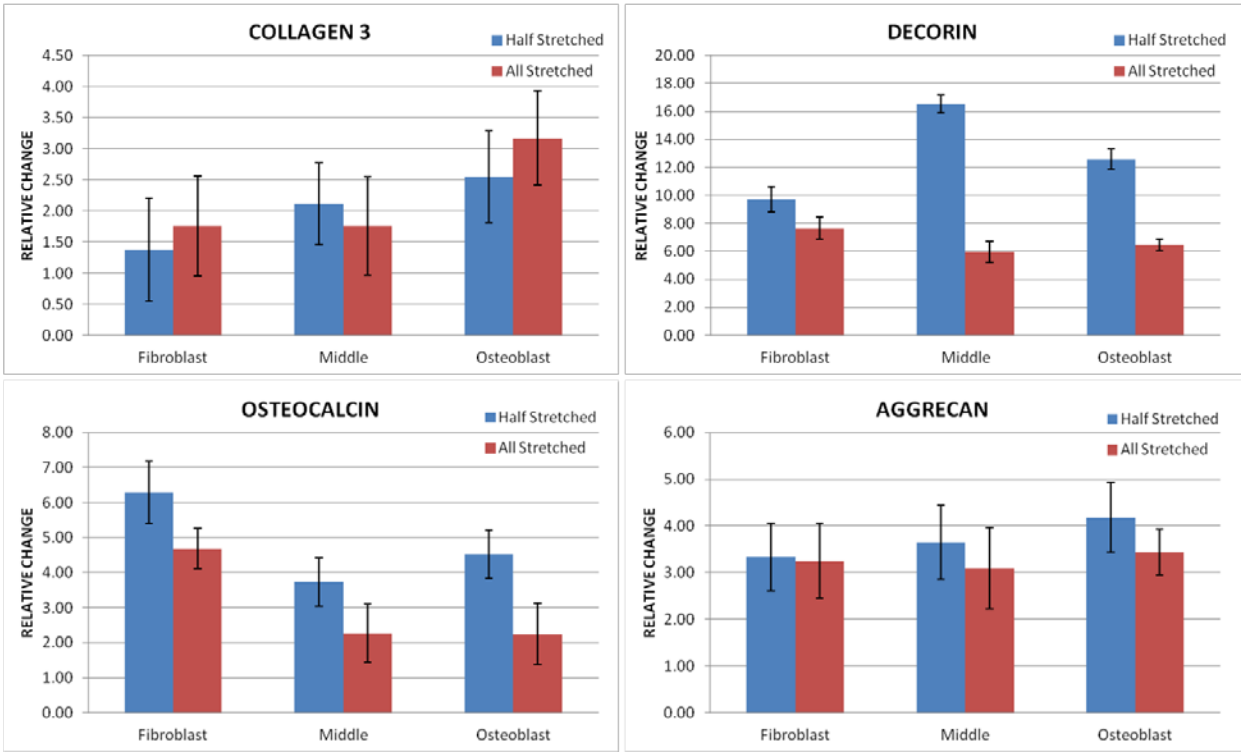

Figure 13. Relative change of MSC gene activation of cells exposed to the different depositions of ECM on scaffolds with mechanical stimulation. Generally the ECM deposited on the all stretched scaffolds activated genes for increased collagen production, decreased mineralization, and more tendon specific GAG deposition with little fibrocartilage deposition.

Task 12: Analyze the ECM deposited on the scaffolds

12.a Each side of the scaffolds will analyzed for total collagen and GAG content (Quarter 6, University of Memphis)

12.b Histology will be performed on the ECM coated scaffolds to visually observe differences in the tendon-side and the bone side of the scaffolds. (Quarter 6, University of Alabama-Birmingham)

The data for Task 12.a is reported in Task 6, as these are duplicate tasks.

Histology was performed at the University of Alabama-Birmingham Center for Metabolic Bone Disease. After scaffolds had been cultured in the mechanical bioreactor and ECM had been deposited across the scaffolds, a half-stretched and all-stretched scaffold was removed from the chamber and frozen. Samples were then fixed in 10% Neutral Buffered Formalin for at least 24 hours, then transferred to 70% for complete fixation. Then all the samples were dehydrated through graded ethanols (80% ETOH X 1, 95% ETOH X 2, and 100% ETOH X 4) to three changes of xylene prior to the infiltration solution (95% Methyl Methacrylate, (MMA), and 5% Dibutyl phthalate, (DBP). Infiltration solutions for all the samples were refreshed every 3 days, for a total of 4 changes. After infiltration, the samples were embedded on edge in a solution composed by 95% MMA and 5% DBP with 0.25% perkodox as the initiator. The samples were then exposed to UV light for polymerization. The fully polymerized (plasticized) sample blocks were trimmed (noting which end was the bone side) and cut to obtain 5um thin sections through the longitudinal axis. Methylene Blue & Basic Fuchsin (H&E like) stain, Goldner's Trichrome stain, Toluidine Blue stain, and Von Kossa stain were then performed.

Images are currently being acquired and analyzed. Histological results will be included in the third manuscript for this project and also the final report to the Army.

MILESTONE #3: Report of animal data (month 18 end of end of 6th quarter)

KEY RESEARCH ACCOMPLISHMENTS

- Established scaffold conditioning protocols for cell attachment.
- Established ECM digestion and analysis protocols that is applicable to multiple scaffold types.
- Selected a single scaffold (PLA fabric) as an appropriate scaffold for tendon/bone interface
- Demonstrated separate co-cultured tissue specific regions on a single scaffold
- Built a custom bioreactor for selective mechanical stimulation in co-culture
- Demonstrated mechanically stimulated ECM activates tissue specific genes in stem cells

REPORTABLE OUTCOMES

This project is expected to have several reportable outcomes. The research will produce three or four manuscript submissions. These will include:

- Scaffold selection for tendon-to-bone tissue engineering (estimated submission date – 11 Jan 2013)
- Fabrication of a co-cultured tissue specific tendon-to-bone scaffold (estimated submission date – 22 Jan 2013)

- Development of a customizable bioreactor for adjustable scaffold stimulation (estimated submission date – FEB 2013)
- Evaluation of a tissue-specific scaffold for tendon-to-bone healing in a rabbit model (estimated submission date – 30 Jan 2013)

This project will also support the fulfillment of two graduate degrees:

- Master's Thesis: Development of a mechanically stimulating bioreactor for tendon-bone tissue engineering (August 2012)
- PhD Dissertation: Tendon-bone tissue engineering (December 2012)

CONCLUSION

The work to this date has pointed out a clear pathway for tissue engineering methods for tendon-to-bone interfaces. Specifically, detailed cell culture techniques and protocols, including cell seeding, ECM digestion and analysis have been established. Based on mechanical testing and ECM deposition on the scaffolds, the best choice as a scaffolding material for this project is the commercially available PLA fabric manufactured by Synthasome, Inc. It was demonstrated that the PLA scaffold could support co-cultured fibroblasts and osteoblasts in separate tissue specific regions on a single scaffold to create a tendon region and bone region. Also a novel custom bioreactor was designed and built for the specific task of stimulation of a tendon directed (fibroblast) end of a scaffold and non-stimulation of a bone directed (osteoblast) end of a scaffold with differing stimulation regimes. The ECM that was deposited on the scaffolds was shown to increase activation of tissue specific genes in rat mesenchymal stem cells compared to tissue culture plastic. Overall, there is promising evidence that this approach would help tendon-to-bone interface repair.

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